

Frequency and Distribution of Tetracycline Resistance Genes in Genetically Diverse, Nonselected, and Nonclinical *Escherichia coli* Strains Isolated from Diverse Human and Animal Sources

Andrew Bryan,^{1†} Nir Shapir,^{1,2,3,4} and Michael J. Sadowsky^{1,2,3*}

Department of Soil, Water, and Climate,¹ BioTechnology Institute,² Center for Microbial and Plant Genomics,³
and Department of Biochemistry, Molecular Biology, and Biophysics,⁴ University of Minnesota,
St. Paul, Minnesota 55108

Received 22 September 2003/Accepted 6 January 2004

Nonselected and natural populations of *Escherichia coli* from 12 animal sources and humans were examined for the presence and types of 14 tetracycline resistance determinants. Of 1,263 unique *E. coli* isolates from humans, pigs, chickens, turkeys, sheep, cows, goats, cats, dogs, horses, geese, ducks, and deer, 31% were highly resistant to tetracycline. More than 78, 47, and 41% of the *E. coli* isolates from pigs, chickens, and turkeys were resistant or highly resistant to tetracycline, respectively. Tetracycline MICs for 61, 29, and 29% of *E. coli* isolates from pig, chickens, and turkeys, respectively, were ≥ 233 $\mu\text{g/ml}$. Multiplex PCR analyses indicated that 97% of these strains contained at least 1 of 14 tetracycline resistance genes [*tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetK*, *tetL*, *tetM*, *tetO*, *tetS*, *tetA(P)*, *tetQ*, and *tetX*] examined. While the most common genes found in these isolates were *tetB* (63%) and *tetA* (35%), *tetC*, *tetD*, and *tetM* were also found. *E. coli* isolates from pigs and chickens were the only strains to have *tetM*. To our knowledge, this represents the first report of *tetM* in *E. coli*.

Problems associated with the presence of antibiotic-resistant bacteria have reached epidemic proportions in recent years, with cost estimates exceeding \$4 billion in the United States alone (6, 12). The spread of antibiotic-resistant bacteria in the environment is dependent on the presence and transfer of resistance genes among microorganisms, mutations, and selection pressure to keep these genes in a population. Selection pressure has been neatly provided by the approximately 50 million pounds of antibiotics that are produced and used each year in the United States (14). Only half of these antibiotics are used for humans, while the remainder are administered to animals or other organisms (8). The causes and effects of antibiotic overuse are varied. One of the most controversial applications of antibiotics, however, is for growth promotion in livestock, and this application has raised concerns about its contribution to the presence of resistant bacteria in humans (1, 25).

Tetracyclines have become the drugs of choice to treat *Mycoplasma*- and *Chlamydia*-induced pneumonia (13) and have been used to treat other atypical pneumonias, rickettsial infections, Lyme disease, ehrlichiosis, and other diseases and cancers (23). The clinically useful chlortetracycline was introduced in 1948 (24). Only a year later, it was shown that young chickens fed tetracyclines had enhanced growth characteristics (10). However, by 1953, it was reported that *Shigella dysenteriae* had developed resistance to tetracycline antibiotics, and by 1955, a

Shigella sp. strain had developed multidrug resistance (20). Because of that history and the broad clinical use of tetracycline, this antibiotic was chosen, along with commensal strains of *Escherichia coli*, to provide a prototypical view of the use of antibiotics and their effects on bacterial populations (21). Tetracycline is a broad-spectrum antibiotic that inhibits bacterial protein synthesis by preventing aminoacyl-tRNA from binding to the bacterial ribosome (20). Resistance to the antibiotic is conferred by 1 or more of the 36 currently described *tet* genes, which encode one of three mechanisms of resistance: an efflux pump, a method of ribosomal protection, or direct enzymatic inactivation of the drug (7). Efflux mechanisms appear to be more abundant among gram-negative microorganisms, while ribosomal protection mechanisms are more common among gram-positive organisms (7). Generally speaking, the rapid spread of tetracycline resistance among bacteria is due to the localization of *tet* genes on plasmids, transposons, and integrons (7, 15, 21).

While several studies have examined tetracycline resistance among bacteria, most have employed clinically isolated bacteria (4, 11, 17) or populations specifically isolated for their ability to grow in the presence of tetracyclines (5, 22). These studies, while useful, do not give an unbiased appraisal of the presence and types of *tet* genes that are present in natural (nonclinical), nonselected populations of bacteria in the environment.

Only a limited number of studies have examined tetracycline resistance determinants in bacteria isolated from a large variety of animal species with different histories of exposure to tetracyclines or in environmental samples (11). While Sengeløv and coworkers (22) examined 100 *E. coli* isolates for the presence of five *tet* resistance determinants and Blake et al. (5) used PCR to examine 200 tetracycline-resistant *E. coli* strains

* Corresponding author. Mailing address: Department of Soil, Water, and Climate, University of Minnesota, 1991 Upper Buford Circle, 439 Borlaug Hall, St. Paul, MN 55108. Phone: (612) 624-2706. Fax: (612) 625-2208. E-mail: sadowsky@umn.edu.

† Present address: Department of Medical Microbiology and Immunology, University of Wisconsin, Madison, WI 53706.

TABLE 1. *E. coli* isolates used in this study and their animal sources

Animal source of <i>E. coli</i>	No. of isolates used for MIC analysis	No. of isolates used for multiplex PCR
Cat	46	9
Cow	158	24
Deer	74	1
Turkey	82	30
Duck	70	1
Human	176	30
Sheep	48	15
Goose	122	3
Dog	47	9
Pig	182	131
Horse	66	3
Chicken	151	66
Goat	41	3
Total	1,263	325

for seven *tet* genes, few have examined a large number of *tet* determinants in nonclinical *E. coli* isolates from a variety of animal species. To better understand the distribution of resistance genes in the environment and to provide insight into selection pressures involved with the use of antibiotics in animal feed, we investigated tetracycline resistance among natural and unselected populations of *E. coli* from 12 animal sources and humans and determined which resistance genes were present in this population.

Isolates and determination of MIC. In order to characterize tetracycline resistance in natural, nonclinical *E. coli* strains from both human and animal sources, 1,263 unique isolates were obtained from humans, cats, cows, deer, turkeys, ducks, sheep, geese, dogs, pigs, horses, chickens, and goats (Table 1). Fecal materials were collected by swabbing the rectal or cloacal region of individual wild and domesticated animals located throughout Minnesota and western Wisconsin as previously described (9). Fecal samples were kept at 4°C and analyzed within 6 h of swabbing. Fecal material was streaked onto mFC agar plates (Difco, BD Diagnostic Systems, Sparks, Md.) and incubated at 44.5°C for 24 h, and six blue colonies from the mFC agar plates were picked and evaluated by using selective and differential growth media as previously described (9). Only isolates giving growth and color responses on all media that

were typical for *E. coli* were used in these studies. Three *E. coli* colonies from each individual fecal sample were used for DNA fingerprinting. All isolates were subjected to DNA fingerprint analysis using rep-PCR and BOXA1R primers (9), and identical clones from the same animal were eliminated from analyses. Unique isolates were grown overnight in 150 µl of Luria-Bertani liquid medium in microtiter plates and were spot inoculated, with a multiple inoculator, onto tryptic soy agar (Difco Laboratories, Detroit, Mich.) supplemented with 0, 5, 10, 20, 40, 70, 93, 117, 175, and 233 µg of tetracycline per ml (Sigma Chemicals, St. Louis, Mo.). The plates were incubated overnight at 37°C and visually examined for growth. MICs were determined from growth patterns, and average values are shown in Fig. 1. If the tetracycline MIC for an isolate was <5 µg/ml, the isolate was considered sensitive to the antibiotic; if it was 10 to 70 or >90 µg/ml, the isolate was considered resistant or highly resistant, respectively. For statistical analysis, a MIC of >233 µg/ml was considered to be 233 µg/ml.

Of the 1,263 *E. coli* isolates examined, 31% were resistant to tetracycline (MICs, >10 µg/ml). Forty-two, 21, 17, and 4% of the isolates from livestock, humans, companion animals (cats, dogs, and horses), and wild animals, respectively, were resistant to tetracycline. More than 78, 47, and 41% of the *E. coli* isolates from pigs, chickens, and turkeys were resistant or highly resistant to tetracycline, respectively. Together these resistant isolates represent about 20% of the 1,263 isolates examined. In contrast, about 22, 30, 3, 3, 21, 33, 7, 23, 6, and 12.2% of the *E. coli* isolates from cats, cows, deer, duck, humans, sheep, geese, dogs, horses, and goats were resistant or highly resistant to tetracycline, respectively. Moreover, the tetracycline MICs for 61, 29, and 29% of *E. coli* isolates from pigs, chickens, and turkeys, respectively, were ≥233 µg/ml. In contrast, the lowest numbers of *E. coli* strains showing resistance or a high level of resistance to tetracycline were those from goats, horses, ducks, geese, and deer. Our results may be explained by the potential exposure of livestock, humans, and companion and wild animals to tetracyclines. Tetracycline is often continuously fed to livestock at subtherapeutic levels for the purpose of growth promotion. For example, up to 70% of U.S. cattle and pig operations use feeds supplemented with antibiotics for growth promotion, and the majority are tetracyclines (2). In contrast, humans and companion animals are most often treated therapeutically, for a limited time, for bac-

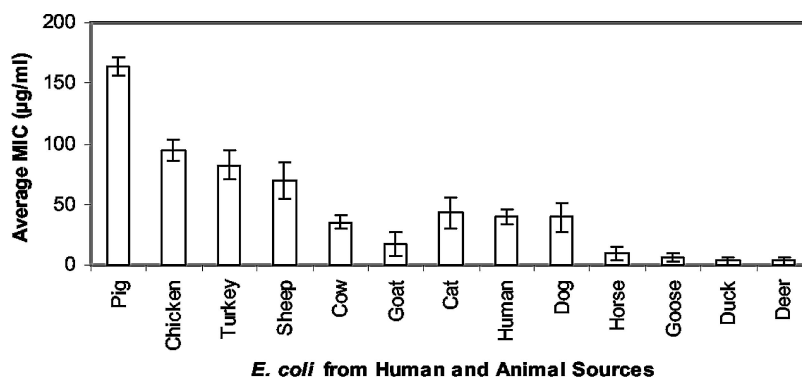


FIG. 1. Average MICs of tetracycline for *E. coli* isolates obtained from pigs, chickens, turkeys, sheep, cows, goats, cats, humans, dogs, horses, geese, ducks, and deer, as determined by the plate dilution method.

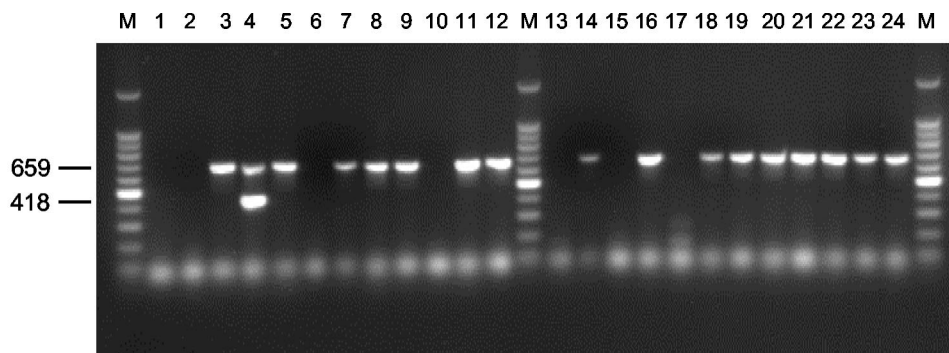


FIG. 2. Representative agarose gel of PCR products from nonclinical *E. coli* isolates, using primer group I, containing primers for *tetB*, *tetC*, and *tetD*. Lanes: 1, no template control; 2, *E. coli* H25; 3, *E. coli* H45; 4, *E. coli* H77; 5, *E. coli* P282; 6, *E. coli* P284; 7, *E. coli* P285; 8, *E. coli* P286; 9, *E. coli* P289; 10, *E. coli* P290; 11, *E. coli* P291; 12, *E. coli* P293; 13, *E. coli* P294; 14, *E. coli* P295; 15, *E. coli* P296; 16, *E. coli* P297; 17, *E. coli* P298; 18, *E. coli* P300; 19, *E. coli* P304; 20, *E. coli* P307; 21, *E. coli* P308; 22, *E. coli* P309; 23, *E. coli* P310; and 24, *E. coli* P312. *E. coli* isolate numbers beginning with P and H were isolated from pigs and horses, respectively. Lane M, molecular weight markers (100 bp ladder). The sizes of the amplicons in base pairs are indicated on the left.

terial infections, perhaps reflecting the intermediate level of resistance to tetracycline (average MICs, 10 to 70 $\mu\text{g/ml}$) of the isolates from these organisms. This resistance level may be changing, however, as other uses of antibiotics become more common, such as the treatment of parasitic and noninfectious diseases (21). The low level of occurrence of tetracycline resistance among isolates from wild animals is presumably due to their low exposure to these antibiotics. Most of these isolates either had a high level of resistance or none at all, suggesting that the acquisition of a mobile genetic element accounts for resistance.

Epidemiology of *tet* genes. All isolates for which the tetracycline MIC was $\geq 93 \mu\text{g/ml}$ (which we considered to indicate a high level of resistance) ($n = 325$) were examined further by use of a multiplex PCR for the presence of the *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetK*, *tetL*, *tetM*, *tetO*, *tetS*, *tetA(P)*, *tetQ*, and *tetX* genes (18). Single-colony isolates were streaked onto plate count agar (Difco), picked using disposable 10- μl sterile loops, and suspended in 50 μl of sterile H_2O . One microliter of the standardized cell suspension served as a template DNA for colony-based multiplex PCR. The primers used for PCR amplification of the 14 tetracycline resistance genes were as described by Ng et al. (18). The primers were aliquoted into four groups: group I contained primers for *tetB*, *tetC*, and *tetD*; group II contained primers for *tetA*, *tetE*, and *tetG*; group III contained primers for *tetK*, *tetL*, *tetM*, *tetO*, and *tetS*; and group IV contained primers for *tetA(P)*, *tetQ*, and *tetX*. PCR was performed with 96-well plates and an MJ Research (Waltham, Mass.) model PTC100 thermocycler, by using the following conditions as described previously (18): 5 min of initial denaturation at 94°C, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min. The PCR products were separated by gel electrophoresis in 1% (wt/vol) agarose gels in 1 \times Tris-acetate-EDTA buffer, stained with ethidium bromide, and visualized under UV illumination. The validity of multiplex PCRs and product sizes was ascertained by using the following positive control plasmids: pSL18, pRT11, pBR322, pSL106, pSL1504, pJA8122, pAT102, pVB.A15, pJ13, pUOA1, pAT451, pJIR39, pNFD13-2, and pBS5, for the genes *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetK*, *tetL*, *tetM*, *tetO*, *tetS*,

tetA(P), *tetQ*, and *tetX*, respectively (18). The sizes of the PCR products were determined by comparison to the migration of a 100-bp ladder (Gibco BRL). The identity of all *tet* genes in a representative sample of nonclinical isolates was ascertained by DNA sequencing of the PCR products, following extraction from agarose gels. A representative agarose gel of PCR products obtained using primer group I, amplifying *tetB*, *tetC*, and *tetD*, is shown in Fig. 2.

Of the 325 strains analyzed by PCR, 97% contained at least 1 of 14 [*tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetK*, *tetL*, *tetM*, *tetO*, *tetS*, *tetA(P)*, *tetQ*, and *tetX*] tetracycline resistance determinants. The most common determinants were Tet B (63% of isolates) and Tet A (35% of isolates) (Fig. 3). However, Tet C, Tet D, and Tet M were also found with various frequencies. The frequencies of *tetA*, *tetB*, *tetC*, and *tetD* in the tested isolates (Fig. 3) were consistent with those previously reported for lactose-fermenting coliforms based on colony hybridization (11). In contrast, Sengeløv and coworkers (22) reported that 71 and 25% of 100 isolates from the diseased and healthy pigs, cattle, and chickens that they tested for five tetracycline resistance determinants contained *tetA* and *tetB*, respectively. None

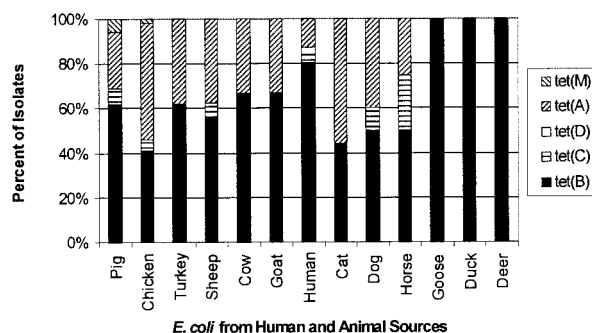


FIG. 3. Frequency of *tetM*, *tetA*, *tetD*, *tetC*, and *tetB* in *E. coli* isolates obtained from pigs, chickens, turkeys, sheep, cows, goats, humans, cats, dogs, horses, geese, ducks, and deer, as determined by colony multiplex PCR. The tetracycline genes *tetE*, *tetG*, *tetK*, *tetL*, *tetO*, *tetS*, *tetA(P)*, *tetQ*, and *tetX* were not found among any of the 325 *E. coli* isolates tested.

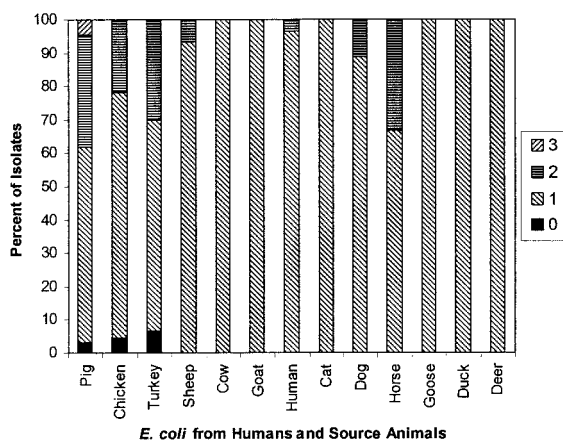


FIG. 4. Percentages of *E. coli* isolates obtained from pigs, chickens, turkeys, sheep, cows, goats, humans, cats, dogs, horses, geese, ducks, and deer, containing multiple tetracycline resistance genes as determined by multiplex PCR using primers for *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetK*, *tetL*, *tetM*, *tetO*, *tetS*, *tetA(P)*, *tetQ*, and *tetX*.

of the tested strains contained *tetE*, *tetG*, *tetK*, *tetL*, *tetO*, *tetS*, *tetA(P)*, *tetQ*, or *tetX*. Since our studies analyzed only highly resistant isolates by PCR, it is possible that additional resistance genes were present in the *E. coli* populations but were nonfunctional or provided only intermediate or low-level resistance.

Isolates from pigs and chickens were the only strains to contain *tetM* and commonly had more than one tetracycline resistance determinant per strain (Fig. 4). The greatest number of strains for which the MICs were high were *E. coli* isolates from these animals. Over 30% of *E. coli* isolates from turkeys, pigs, and horses contained two tetracycline resistance determinants, and 4.5% of the pig isolates contained three *tet* genes. However, the presence of more than one resistance determinant did not lead to noticeably higher MICs. It is possible that strong selection pressures provided by environments containing elevated levels of tetracycline lead to the acquisition of more than one tetracycline gene in a given strain due to their prevalence in the environment, rather than to a selective advantage. The results of our studies also showed that 22.2 and 1.9% of the isolates contained two and three *tet* genes, respectively. This is in contrast to results from previous studies, in which only 3.5% (16) and 5.4% (22) of isolates had two genes, perhaps due to our use of a larger number and variety of isolates and to the greater number of genes examined.

To our knowledge, this is the first report documenting the presence of the *tetM* gene in *E. coli* (7). Due to the uniqueness of these results, the presence of *tetM* in one of our *E. coli* isolates from pigs was verified by sequencing the PCR product produced using *tetM*-specific primers. BLAST analysis (3) indicated that of the 386 bp of high-quality and continuous sequence examined, there was 98% nucleotide sequence identity to the *tetM* gene from *Enterococcus faecalis* (GenBank accession number M85225). The *tetM* gene, which imparts resistance to tetracyclines by encoding a ribosomal protection mechanism, commonly occurs in transposons Tn916 and Tn1545. The *tetM* gene is widely dispersed among various gram-positive organisms, but it has only rarely been docu-

mented in gram-negative bacteria (19, 21). The presence of *tetM* in *E. coli* is most likely due to genetic transfer from *Enterococcus*, a common carrier of *tetM* (8). Evidence for this possibility is provided by the studies of Poyart et al. (19), who demonstrated the in vitro transfer of Tn916 from *E. faecalis* to *E. coli* (16).

In summary, by examining the frequency and distribution of tetracycline resistance genes among diverse natural *E. coli* populations present in different animal species, a picture of the selection pressures in the various host animals can be inferred. Not only did those animal hosts that presumably had continuous exposure to tetracycline have a higher percentage of tetracycline-resistant *E. coli* isolates, but also those isolates carried a greater diversity of resistance genes. Moreover, these isolates often had more than one tetracycline resistance determinant and contained a *tet* gene previously thought not to be present in *E. coli*. This suggests that human activity provides environments that select for resistant strains and encourages the transfer of genetic information from unrelated bacterial species. Although this study examined only nonclinical *E. coli* isolates, the prevalence of tetracycline resistance genes among these unrelated bacteria, and circumstantial and direct evidence of horizontal gene transfer, suggests that these same resistance determinants may also be present in animal and human pathogens.

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